EXHIBIT A

Expressi n cl ning f GABA$_B$ receptors uncovers similarity t_ metabotropic glutamate receptors

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GABA (γ-amino-butyric acid), the principal inhibitory neurotransmitter in the brain, signals through ionotropic (GABA$_A$/GABA$_C$) and metabotropic (GABA$_B$) receptor systems. Here we report the cloning of GABA$_B$ receptors. Photoaffinity labelling experiments suggest that the cloned receptors correspond to two highly conserved GABA$_B$ receptor forms present in the vertebrate nervous system. The cloned receptors negatively couple to adenylyl cyclase and show sequence similarity to the metabotropic receptors for the excitatory neurotransmitter L-glutamate.

The majority of synapses in the central nervous system (CNS) use either GABA or L-glutamate as neurotransmitters to control neuronal inhibition and excitation. GABA, like other neurotransmitters including L-glutamate, serotonin and acetylcholine, activates both ionotropic and metabotropic receptors[1–8]. The ionotropic receptors are ligand-gated ion channels that convey fast synaptic transmission. In contrast, metabotropic receptors couple to G proteins (guanine-nucleotide-binding proteins) and modulate synaptic transmission through intracellular effector systems. Molecular cloning has revealed that the ionotropic receptors for L-glutamate and GABA belong to two separate gene families[2,5]. The metabotropic receptors for L-glutamate (mGluRs) differ structurally from other 7TM G-protein-coupled neurotransmitter receptors[9] and constitute a new gene family[7,8]. The molecular structure of metabotropic GABA$_B$ receptors, first reported in 1981[10], has remained elusive.

GABA$_B$ receptors modulate synaptic transmission by presynaptic inhibition of transmitter release or by increasing a K^+-conductance responsible for long-lasting inhibitory postsynaptic potentials (late IPSPs)[1,3,4,6,11]. Presynaptic and postsynaptic GABA$_B$ receptor subtypes were proposed[12–16]. Presynaptically, GABA$_B$ autoreceptors have been described controlling the release of GABA, whereas GABA$_B$ heteroreceptors regulate the release of L-glutamate, noradrenaline, dopamine, 5-hydroxytryptamine, substance P, cholecystokinin or somatostatin. The induction of long-term potentiation, an associative increase in synaptic strength that may underlie the formation of some types of learning and memory, is affected by the activation of pre- and postsynaptic GABA$_B$ receptors[17,18]. Baclofen (Lioresal), a GABA$_B$ receptor agonist introduced onto the market in 1972, is used to treat spasticity following multiple sclerosis and spinal injury[1,6]. Since then, other potential clinical applications, including absence epilepsy, anxiety, depression and cognition deficits, have become apparent[3,4]. Given the physiological and clinical importance, many attempts to characterize GABA$_B$ receptors at the molecular level have been made, but as yet have met with only limited success. The purification of a putative GABA$_B$ receptor protein of relative molecular mass 80,000 (M_r 80K) was reported[19], but no amino-acid sequence was disclosed.

With the aim of isolating a GABA$_B$ receptor complementary DNA using expression cloning, we designed a new high-affinity GABA$_B$ receptor antagonist. This ligand allowed the identification of cDNAs encoding two GABA$_B$ receptor proteins, designated GABA$_B$R1a and -b. Amino-acid sequence analysis of the cloned receptors revealed that the metabotropic receptors for GABA and L-glutamate comprise a gene family. Using a ^{125}I-labelled photoaffinity derivative of the new antagonist, we detected two GABA$_B$ receptor proteins of 130K and 100K in the CNS. These GABA$_B$ proteins are expressed in species as different as man and fish, indicating conservative evolution among vertebrates. The cloned GABA$_B$R1a receptor negatively couples to adenylyl cyclase when stably expressed in HEK293 cells. Recombinantly expressed GABA$_B$R1a and -b proteins have similar M_rs and pharmacology to native receptors. Their transcripts are abundant and expressed in all major brain structures. Taken together, this suggests that the cloned receptors correspond to the two GABA$_B$ receptor forms that can be photoaffinity labelled in the CNS.

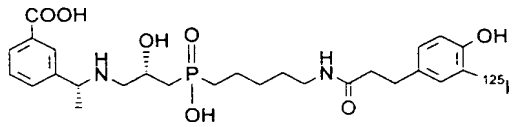
GABA$_B$ receptor-specific radioligands

The agonist affinity, but not the antagonist affinity, depends on the G-protein coupling status of GABA$_B$ receptors[20]. For expression cloning of GABA$_B$ receptors using a radioligand binding assay we therefore aimed at developing a high-affinity antagonist. With [^{125}I]CGP64213, we introduce a ligand with the highest affinity so far described (K_d = 1.2 ± 0.2 nM) for the GABA$_B$ binding site (Fig. 1). Based on the same structure we designed a photoaffinity ligand, [^{125}I]CGP71872 (K_d = 1.0 ± 0.2 nM), which can be crosslinked to the receptor protein (Fig. 1). Both radioligands demonstrate antagonist activity at pre- as well as postsynaptic GABA$_B$ receptors, as shown by complete suppression of L-baclofen-induced responses in electrophysiological recordings from rat CA1 hippocampal slices (M. F. Pozza, personal communication). In competition binding experiments using rat cortical cell membranes the radioligands exhibit high selectivity for GABA$_B$ receptors (Fig. 2b and see Fig. 6). Neither L-glutamate nor compounds selective for ionotropic GABA$_A$[5] or GABA$_C$[21] receptors or the GABA uptake system displaced [^{125}I]CGP64213 or [^{125}I]CGP71872 from cortical GABA$_B$ receptors (Fig. 2b and data not shown). CGP64213 or CGP71872 have no effect in binding assays for kainate, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) (agonist and co-agonist sites), muscarinic, α_1-/α_2-adrenergic, β-adrenergic, 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, histamine$_1$, histamine$_2$, adenosine$_1$, μ-opiate and substance P receptors at concentrations of up to 1 μM (data not shown).

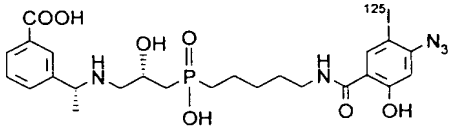
Labelling of native GABA$_B$ receptors

[^{125}I]CGP71872 photoaffinity labelling of cortex, cerebellum and spinal cord cell membranes reveals two putative GABA$_B$ proteins of 130K and 100K (Fig. 2a). These data are in contrast to previous

studies suggesting a M_r of 80K for $GABA_B$ receptors[19]. The M_rs rule out similarity with $GABA_A$/$GABA_C$ receptors and the GABA uptake system. The two putative $GABA_B$ proteins are differentially expressed in the nervous system. In cerebellum the 100K protein is predominant over the 130K protein, whereas in spinal cord the 130K protein is more prevalent. In cortex tissue both proteins appear equally abundant. No proteins are labelled in tissues such as liver and kidney expected to lack $GABA_B$ receptors. Native $GABA_B$ receptors were photoaffinity labelled in the presence of various competitor substances (Fig. 2b). Neither the $GABA_A$ selective ligands muscimol and bicuculline nor the $GABA_C$ receptor agonist



[^{125}I]CGP 64213



[^{125}I]CGP 71872

Figure 1 Chemical structures of the $GABA_B$ receptor-specific ligands [^{125}I]CGP64213 and [^{125}I]CGP71872.

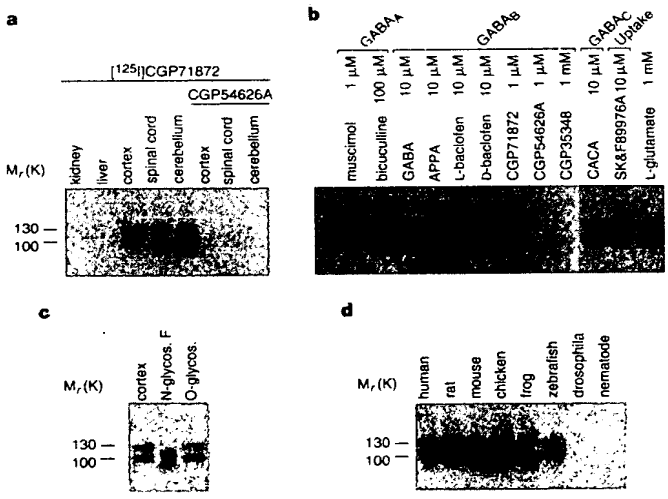


Figure 2 Photoaffinity crosslinking of $GABA_B$ receptor proteins. Cell membranes of the tissues indicated were photoaffinity labelled with [^{125}I]CGP71872 and subjected to SDS-PAGE and autoradiography. **a**, **b**, Selectivity of the photoaffinity ligand [^{125}I]CGP71872. **a**, Differential distribution of $GABA_B$ receptor variants of 130K and 100K in tissues of the nervous system. [^{125}I]CGP71872 binding is inhibited by addition of 1 μM of CGP54626A, a selective $GABA_B$ receptor antagonist[24]. **b**, Competition of [^{125}I]CGP71872 labelling by different ligands. Incubation of membrane extracts with the photoaffinity ligand was carried out in the presence of competitor substances at the concentrations indicated. **c**, $GABA_B$ receptors are *N*-glycosylated. Photoaffinity-labelled rat cortex cell membranes were incubated with 0.4 units *N*-glycosidase F or 0.6 milliunits *O*-glycosidase (Boehringer Mannheim). **d**, Photolabelling of $GABA_B$ receptors from different species. Brain tissues from the species indicated were labelled as described in Methods. In the case of *Drosophila melanogaster* and *Haemonchus concortus* whole animals were analysed.

cis-aminocrotonic acid (CACA) or the inhibitor of the GABA uptake system, SK&F89976A[22], compete significantly for radioligand binding. In contrast, the $GABA_B$ receptor agonists GABA, 3-aminopropylphosphinic acid (APPA) and L-baclofen compete with [^{125}I]CGP71872 for binding. As another known criterion[23], L-baclofen competes more potently than D-baclofen. The $GABA_B$ receptor antagonists CGP54626A[24], CGP35348[24] and the non-radioactive photoaffinity ligand are also effective displacers of [^{125}I]CGP71872 at native receptors. For all ligands tested, there is no visible difference in the displacement of [^{125}I]CGP71872 at the 130K and 100K proteins, indicating a qualitatively similar binding pharmacology for the two receptors.

Native $GABA_B$ receptors are *N*-glycosylated, as shown by the reduction in M_r to 110K and 90K, respectively, after cleavage with *N*-glycosidase F (Fig. 2c). No significant shift in M_r is detected after enzymatic treatment with *O*-glycosidase (Fig. 2c). Photoaffinity-labelled proteins of 130K and 100K are detectable in tissues from all vertebrate species analysed, including zebrafish (Fig. 2d), suggesting that the two proteins and their antagonist binding sites are highly conserved. The avian $GABA_B$ receptor proteins have M_rs slightly higher than in other species, possibly reflecting differences in glycosylation and/or RNA splicing. No binding of the photoaffinity ligand to any protein could be detected in the fruitfly *Drosophila melanogaster* or the nematode *Haemonchus concortus*.

Expression cloning of a $GABA_B$ receptor cDNA

As with many neurotransmitter receptors, biochemical isolation of $GABA_B$ receptors was hindered by the lack of (1) ligands that bind under solubilizing conditions with high affinity to the protein and (2) cell lines that express significant amounts of receptor protein. We therefore explored cloning strategies using *Xenopus* oocytes and electrophysiological measurements, without success. To overcome these problems, we used an expression cloning procedure in which we screened for [^{125}I]CGP64213 binding at transfected COS-1 cells.

A high density of $GABA_B$ receptor binding sites is found in cortex and cerebellum of 7-day-old rats[25]. Those tissues were therefore used as a source to construct a cDNA library (complexity 2×10^6 clones) in the expression vector pcDNAI. The library was divided into pools of 2,000 cDNA clones each and individual pools were transfected into COS-1 cells and screened for radioligand binding. A total of 310 pools were analysed until one positive pool and, after serial subdivisions, a single clone containing a 4.4 kilobase (kb) cDNA insert was identified. This cDNA clone encodes a protein of 960 amino acids, designated $GABA_BR1a$ (Fig. 3a). When expressed in COS-1 cells the $GABA_BR1a$ protein has an apparent M_r that is similar to that of the 130K $GABA_B$ receptor present in brain, as shown by photoaffinity labelling (Fig. 3c). The amino-terminal 16 amino acids of $GABA_BR1a$ probably constitute the signal peptide[26]. The calculated M_r of the mature protein is 106K, similar to the M_r of the native 130K $GABA_B$ receptor protein after treatment with *N*-glycosidase F (Fig. 2c). Consistent with *N*-glycosylation of native $GABA_B$ receptors, $GABA_BR1a$ contains several consensus *N*-glycosylation sites in the N-terminal extracellular domain (Fig. 3a). Putative substrate sites[27] for casein kinase II and protein kinase C are found in cytoplasmic domains, suggesting that receptor activity could be regulated by phosphorylation.

We screened the cDNA library by low-stringency hybridization using the $GABA_BR1a$ cDNA as a probe. Several clones distinct from the $GABA_BR1a$ cDNA were isolated. A 2.9 kb cDNA encodes a protein of 844 amino acids, designated $GABA_BR1b$ (Fig. 3a). The mature $GABA_BR1b$ differs from $GABA_BR1a$ in that the N-terminal 147 residues are replaced by 18 different residues (Fig. 3a). Presumably, the $GABA_BR1a$ and -b receptor variants are derived from the same gene by alternative splicing. Transient expression of the $GABA_BR1b$ cDNA in COS-1 cells yields a protein with a similar M_r to the 100K receptor detected in brain tissue (Fig. 3c). The calculated M_r of the mature $GABA_BR1b$ is 92K, similar to that of

the deglycosylated 100K brain receptor (Fig. 2c). Northern blot analysis (Fig. 4) indicates that transcripts for both $GABA_BR1a$ and -b are abundant in brain and neither represent cloning artefacts nor rare aberrant splice events. In support of this, we found (1) several full-length $GABA_BR1a$ and -b clones in rat brain and spinal cord cDNA libraries and (2) several partial human $GABA_BR1a$ and -b cDNA clones (data not shown).

$GABA_B$ receptors and mGluRs

The $GABA_BR1a/b$ proteins are considerably larger than classic G-protein-coupled receptors[9] and are similar in size to mGluRs[7,8] ranging from 872 to 1,203 amino-acid residues. Indeed, Blast[28] and FASTA[29] database searches indicate that $GABA_BR1$ is most closely related to members of the mGluR gene family which contains eight subtypes as well as a Ca^{2+}-sensing receptor[30]. Bestfit[27] sequence alignments of $GABA_BR1a$ with these receptors reveals 18–23% amino-acid sequence identity and 43–48% related residues (Fig. 3a and data not shown). The similarity between $GABA_BR1a$ and the mGluRs is in the same range as for distant subunits of the ionotropic glutamate receptor gene family[2]. Although the amino-acid sequence identity between $GABA_BR1a$ and the mGluRs is low, the conservation of the structural architecture is clearly evident from the hydrophobicity profiles[31] (Fig. 3b). As for the mGluRs, a large N-terminal extracellular domain precedes seven closely spaced putative transmembrane domains, indicative of G-protein-coupled receptors. The sequence similarity of $GABA_BR1$ to individual mGluRs is not confined to specific domains; notably it is not restricted to the transmembrane regions (Fig. 3a). A total of 208 amino-acid residues are conserved in all mGluRs and 27 of these are preserved in $GABA_BR1$ (Fig. 3a; empty circles), further emphasizing that $GABA_B$ receptors and mGluRs are related. Twenty-one cysteine residues are conserved in all mGluRs (Fig. 3a; filled circles) and are considered a hallmark of the mGluR gene family. Most cysteine residues are not conserved in $GABA_BR1$. Nine cysteines are closely spaced in the N-terminal extracellular domain of mGluRs, a part of the protein referred to as the cysteine-rich region. Such a region is missing in $GABA_BR1$. Blast and FASTA database searches with the $GABA_BR1$ sequence also reveal weak similarities to guanylyl cyclases, that is, the natriuretic peptide receptor[32], to selectins[33] and to the complement receptor type 1[34], as well as to bacterial amino-acid-binding proteins[35,36]. No significant sequence similarity is found to $GABA_A$ or $GABA_C$ receptors nor to G-protein-coupled receptors[9] other than the mGluRs.

In their N-terminal extracellular domain, mGluRs contain two lobes with structural similarity to the amino-acid binding sites of bacterial proteins[37]. It has been proposed that these lobes constitute the L-glutamate binding site of mGluRs. A FASTA search reveals that a structural similarity to bacterial amino-acid binding proteins is also evident for the N-terminal extracellular domain of $GABA_BR1$ (Fig. 3a; arrows). Strikingly, the N-terminal extracellular domain of the smaller $GABA_BR1b$ receptor is limited to the region with structural similarity to the bacterial proteins.

It was shown for mGluRs that the second intracellular loop (Fig. 3a; ICL2) determines the specificity of G-protein coupling[38]. In this region, $GABA_BR1$ does not show any significant sequence similarity to mGluRs. Therefore one of several possible signal transduction pathways, as described for mGluRs[7,8], cannot be inferred. However, as in the mGluRs, the ICL1 and ICL3 are small and the ICL2 region is rich in basic residues and therefore is expected to be involved in G-protein interaction.

Spatial distribution of $GABA_BR1$ transcripts

The tissue distribution of $GABA_BR1a$ and -b mRNA was examined by northern blot analysis (Fig. 4) and *in situ* hybridization (Fig. 5). Northern blots hybridized with a probe containing sequences common to both $GABA_BR1a$ and -b (Fig. 4a; pan probe) revealed RNAs of 4.3 to 4.4 kb and 3 kb present in brain and testis, but not in the other tissues analysed (Fig. 4b). The size of these transcripts indicates that the cloned 4.4 kb $GABA_BR1a$ cDNA represents a nearly full-size mRNA. Separate analysis of $GABA_BR1a$ and -b transcripts (Fig. 4a; probes R1a and R1b) revealed RNAs of 4.4 kb and ~1.8 kb for R1a and RNAs of 4.3 kb and 3 kb for R1b, clearly demonstrating the expression of both receptor variants in brain (Fig. 4b). The sequences of the 3 kb and 1.8 kb transcripts are unknown, but the 1.8 kb RNAs appear too short to encode a functional $GABA_B$ receptor.

In situ hybridization studies were carried out using the pan probe that does not hybridize to the 1.8 kb RNAs (Fig. 5). $GABA_BR1$ receptor transcripts are abundant in all cerebral cortical layers, in the pyramidal cell layers of the hippocampus, the granular cell layer of the dentate gyrus (Fig. 5a, b) and in the basal ganglia (data not shown), including the caudate putamen, nucleus accumbens and olfactory tubercle. In the cerebellum, transcripts are found in abundance in the Purkinje cells and at moderate levels in the

Table 1 Binding affinities of native and recombinant $GABA_B$ receptors

	Rat cerebral cortex		$GABA_BR1a$		$GABA_BR1b$	
Agonists	IC_{50} (μM)	Hill coeff. n_H	IC_{50} (μM)	Hill coeff. n_H	IC_{50} (μM)	Hill coeff. n_H
GABA	0.14 ± 0.03	0.55 ± 0.03	22.9 ± 2.4	0.84 ± 0.04	31.1 ± 4.0	0.89 ± 0.06
APPA	0.018 ± 0.001	0.57 ± 0.02	2.3 ± 0.5	0.75 ± 0.06	2.8 ± 0.2	0.81 ± 0.06
L-baclofen	0.21 ± 0.05	0.54 ± 0.04	25.1 ± 7.8	0.74 ± 0.14	35.1 ± 4.2	0.80 ± 0.02
CGP47656	0.29 ± 0.08	0.74 ± 0.04	10.8 ± 2.2	0.73 ± 0.11	15.0 ± 2.4	0.79 ± 0.10
Antagonists						
CGP56999A	0.0004 ± 0.0001	1.17 ± 0.06	0.0007 ± 0.0002	1.14 ± 0.13	0.0008 ± 0.0001	0.99 ± 0.05
CGP62349	0.0007 ± 0.0001	1.07 ± 0.06	0.0011 ± 0.0002	0.87 ± 0.03	0.0016 ± 0.0008	0.88 ± 0.05
CGP64213	0.0016 ± 0.0003	1.08 ± 0.02	0.0023 ± 0.0001	1.01 ± 0.05	0.0031 ± 0.0001	1.00 ± 0.04
CGP54626A	0.0022 ± 0.0005	0.99 ± 0.02	0.0015 ± 0.0001	0.88 ± 0.03	0.0020 ± 0.0001	1.01 ± 0.05
CGP71872	0.0024 ± 0.0008	1.08 ± 0.03	0.0036 ± 0.0004	1.05 ± 0.18	0.0051 ± 0.0004	1.02 ± 0.07
CGP35348	4.2 ± 0.8	0.85 ± 0.02	17.2 ± 3.1	0.67 ± 0.06	21.7 ± 1.3	0.81 ± 0.09
2-OH-saclofen	12.4 ± 1.1	0.84 ± 0.04	82.7 ± 10.5	0.85 ± 0.06	87.5 ± 7.9	0.74 ± 0.04
Saclofen	28.3 ± 1.9	0.81 ± 0.04	290.0 ± 35.3	0.73 ± 0.04	366.0 ± 32.2	0.65 ± 0.10
SCH50911	0.27 ± 0.02	0.96 ± 0.02	0.38 ± 0.03	0.87 ± 0.01	0.43 ± 0.04	0.78 ± 0.04

Inhibition of [^{125}I]CGP64213 binding to native and recombinant $GABA_B$ receptors by $GABA_B$ agonists and antagonists. Rat cerebral cortex membranes and membranes from COS-1 cells transiently transfected with the $GABA_BR1a$ and -b cDNAs were used. IC_{50} values and Hill coefficients were fitted using nonlinear regression (PRISM program, Graph Pad Software Inc., San Diego). Values are means ± s.e.m. of 3 independent experiments; APPA, 3-aminopropylphosphinic acid.

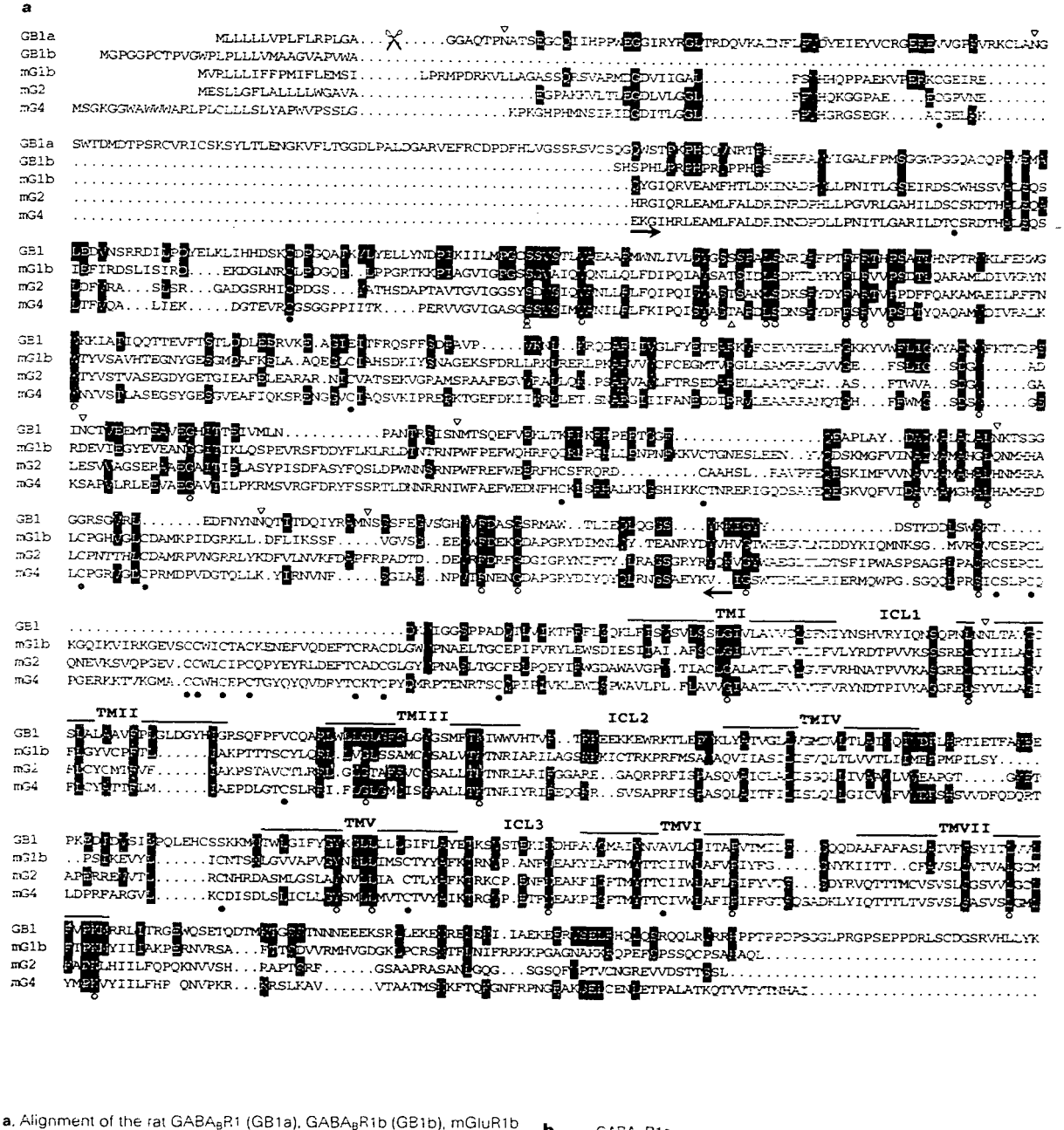


Figure 3 a, Alignment of the rat GABA$_B$R1 (GB1a), GABA$_B$R1b (GB1b), mGluR1b (mG1b), mGluR2 (mG2) and mGluR4 (mG4) amino-acid sequences. The sequences of GABA$_B$R1a and GABA$_B$R1b differ at the N terminus and are otherwise identical. Amino acids that are identical in the N-terminal sequences of GABA$_B$R1a and GABA$_B$R1b and amino acids that are conserved among GABA$_B$R1 and one or more of the three mGluRs are boxed in black. The putative transmembrane domains (TMI-TMVII) and intracellular loops (ICL1-ICL3) are indicated. Proposed signal peptide cleavage sites[26] are marked with scissors. Putative *N*-glycosylation sites are found at amino-acid positions 7, 67, 392, 423, 465, 485, 497 and 614 of the predicted mature GB1a and are marked by inverted triangles. Residues conserved throughout the mGluRs and GABA$_B$R1 are indicated by empty circles, conserved cysteines of mGluRs are denoted by filled circles. Residues in mGluR1[37] affecting L-glutamate affinity are indicated by triangles, arrows delimit the region where GABA$_B$R1 has structural similarity with bacterial amino-acid binding proteins. **b**, Hydropathy profiles of the GABA$_B$R1 and mGluR1b sequences, computed according to Kyte and Doolittle[27] using sequence analysis programs from the University of Wisconsin Genetics Computer Group[27]. Black colouring indicates the positions of the hydrophobic transmembrane domains, grey colouring the N-terminal signal peptides. **c**, [^{125}I]CGP71872 photoaffinity labelling of cell membranes from rat cortex and COS-7 cells transiently transfected with the GABA$_B$R1a and GABA$_B$R1b cDNA. Autoradiography of a SDS-PAGE (6%) is shown. Recombinant GABA$_B$ receptor proteins sometimes appear as a doublet band in SDS-PAGE, probably due to photoaffinity labelling of incompletely processed receptors.

granular layer (Fig. 5d, e). $GABA_BR1$ mRNAs are expressed in neuronal but not glial cells (for example, Fig. 5c). A direct comparison of the distribution of transcripts and protein in the CNS is difficult. There is a close overlap of $GABA_BR1$ mRNA and $GABA_B$ receptor binding sites, as assessed by autoradiography using radioligands[39,40], in the medial habenula (Fig. 5a), the medial geniculate nucleus (Fig. 5b) and the interpeduncular nucleus. In other CNS areas the regional abundance of transcripts and binding sites differs. In the cerebral cortex transcript levels are higher in layer VIb than in layers II–V, whereas the opposite has been reported for the density of receptor binding sites[39,40]. In the hippocampal formation, expression of the mRNA is detected in the pyramidal and granule cell layers, whereas receptor binding sites were reported in the molecular layers. In the cerebellum, transcripts are found in the Purkinje cells and in the granular layer (Fig. 5d, e), whereas $GABA_B$ receptor binding sites were reported to be most abundant in the molecular layer, but also to be present in the granular layer[39,40]. In the cerebellum $GABA_B$ receptors may therefore be located on Purkinje cell dendrites and granule cell parallel fibres, in agreement with previous studies[39–41].

Pharmacological profiles

To compare the pharmacological profiles of $GABA_BR1a$/-b expressed in mammalian cells (COS-1/HEK293) and $GABA_B$ receptors present in rat cerebral cortex, we analysed the inhibition of [^{125}I]CGP64213 binding by selected $GABA_B$ receptor antagonists and agonists (Fig. 6a, b; Table 1). The inhibition curves for the potent $GABA_B$ receptor antagonists CGP56999A[24] and CGP54626A[24] at native and recombinant receptors are almost identical (Fig. 6a). For all potent antagonists tested the 50%

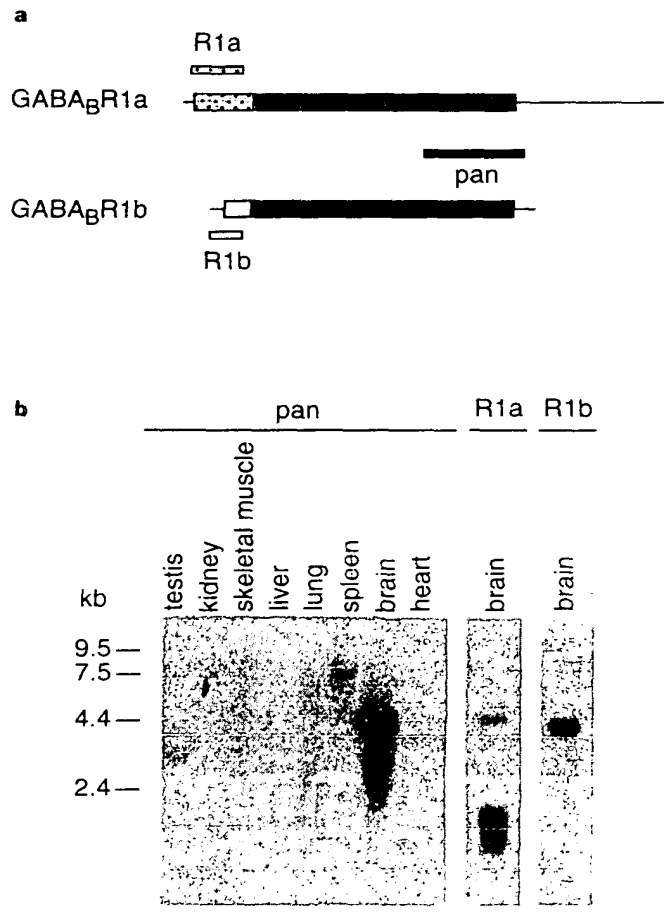


Figure 4 Northern blot analysis of $GABA_BR1a$ and -b mRNA expression. **a**, Schematic diagram showing the localization of the probes used. Coding sequences are boxed, untranslated sequences are indicated by a line. Only N-terminal sequences of $GABA_BR1a$ and -b differ. The R1a probe corresponds to nucleotides 1 to 405 of the $GABA_BR1a$ cDNA, the R1b probe to nucleotides 16 to 259 of the $GABA_BR1b$ cDNA. The pan probe is common to both $GABA_BR1a$ and -b and is derived from nucleotides 2,462 to 3,234 of the $GABA_BR1a$ cDNA. **b**, Rat multiple tissue northern blots (Clontech, Palo Alto) with 2 μg poly(A)$^+$ RNA per lane were hybridized to random-primed ^{32}P-labelled probes.

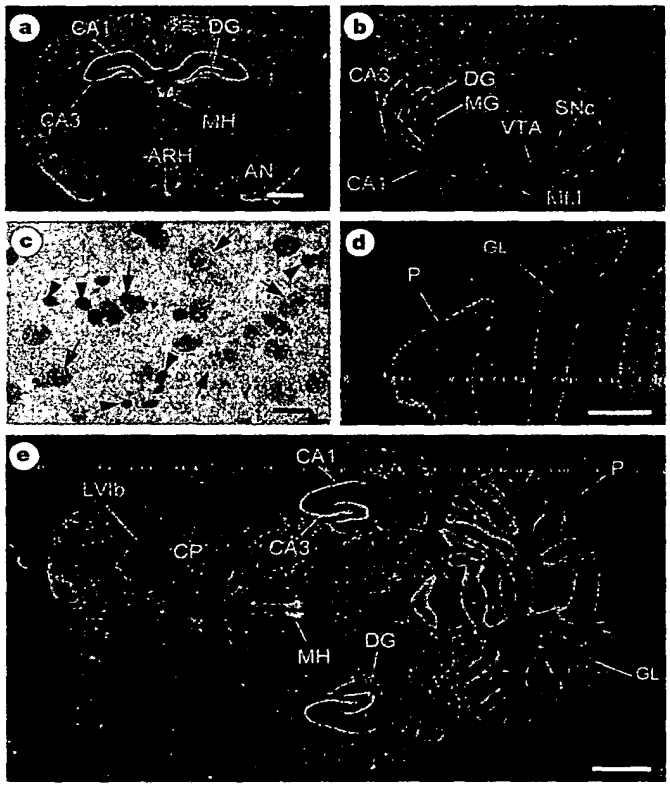


Figure 5 *In situ* hybridization analysis of $GABA_BR1$ transcripts in rat brain. Tissue sections were hybridized to ^{35}S-labelled antisense probes. Darkfield illuminations of representative autoradiograms of coronal (**a**, **b**, **d**) and horizontal sections (**e**), and a brightfield illumination (**c**) are shown. **a**, Dorsal hippocampus plane; **b**, ventral hippocampus plane; **c**, CA3 field of the hippocampus; **d**, lobules of the cerebellar cortex; **e**, dorsal tier of the brain. Transcripts are abundant in all cerebral cortical layers, especially in the layer VIb (LVIb), in the pyramidal cell layer of the CA1-CA3 subfields of the hippocampus as well as in the granular layer of the dentate gyrus (DG) and in the medial habenula (MH). $GABA_BR1$ mRNA is detected in the medial geniculate nucleus (MG), in the substantia nigra, pars compacta (SNc), in the ventral tegmental area (VTA) and in several thalamic, amygdaloid (AN) and hypothalamic nuclei, such as the arcuate nucleus of the hypothalamus (ARH) and mammillary bodies of the hypothalamus (MM). In the cerebellum, high levels of transcripts are found in the Purkinje cells (P) and moderate levels in the granular layer (GL). Arrows indicate neuronal, arrowheads glial cells. The sections were exposed to nuclear emulsion for 12 days. No hybridization signal was observed with radiolabelled sense probes. Scale bars, 2 mm (**a**, **b**), 10 μM (**c**), 30 μM (**d**), 1.3 mm (**e**).

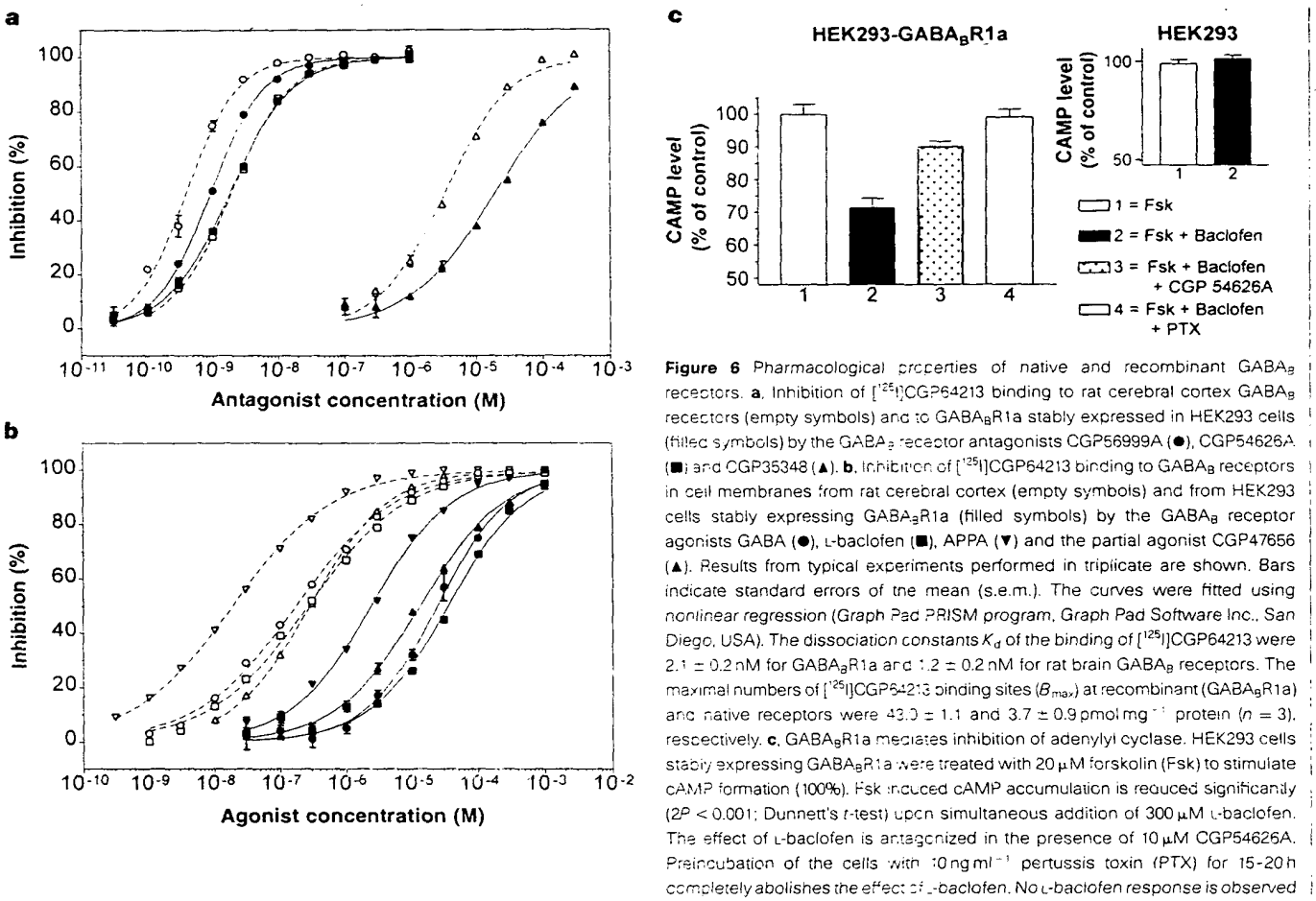


Figure 6 Pharmacological properties of native and recombinant $GABA_B$ receptors. **a**, Inhibition of [^{125}I]CGP64213 binding to rat cerebral cortex $GABA_B$ receptors (empty symbols) and to $GABA_BR1a$ stably expressed in HEK293 cells (filled symbols) by the $GABA_B$ receptor antagonists CGP56999A (●), CGP54626A (■) and CGP35348 (▲). **b**, Inhibition of [^{125}I]CGP64213 binding to $GABA_B$ receptors in cell membranes from rat cerebral cortex (empty symbols) and from HEK293 cells stably expressing $GABA_BR1a$ (filled symbols) by the $GABA_B$ receptor agonists GABA (●), L-baclofen (■), APPA (▼) and the partial agonist CGP47656 (▲). Results from typical experiments performed in triplicate are shown. Bars indicate standard errors of the mean (s.e.m.). The curves were fitted using nonlinear regression (Graph Pad PRISM program, Graph Pad Software Inc., San Diego, USA). The dissociation constants K_d of the binding of [^{125}I]CGP64213 were 2.1 ± 0.2 nM for $GABA_BR1a$ and 1.2 ± 0.2 nM for rat brain $GABA_B$ receptors. The maximal numbers of [^{125}I]CGP64213 binding sites (B_{max}) at recombinant ($GABA_BR1a$) and native receptors were 43.0 ± 1.1 and 3.7 ± 0.9 pmol mg^{-1} protein (n = 3), respectively. **c**, $GABA_BR1a$ mediates inhibition of adenylyl cyclase. HEK293 cells stably expressing $GABA_BR1a$ were treated with 20 μM forskolin (Fsk) to stimulate cAMP formation (100%). Fsk-induced cAMP accumulation is reduced significantly ($2P < 0.001$; Dunnett's t-test) upon simultaneous addition of 300 μM L-baclofen. The effect of L-baclofen is antagonized in the presence of 10 μM CGP54626A. Preincubation of the cells with 100 ng ml^{-1} pertussis toxin (PTX) for 15–20 h completely abolishes the effect of L-baclofen. No L-baclofen response is observed in non-transfected HEK293 cells (inset). Bars represent mean values + s.e.m. of at least three independent experiments performed in quadruplicate.

inhibitory concentration (IC_{50}) values are similar for native and cloned $GABA_B$ receptors (Table 1). The Hill coefficients are close to 1, indicating single high-affinity binding sites. Low-affinity $GABA_B$ receptor antagonists, such as CGP35348[24], saclofen[4] and 2-OH-saclofen[4], have 4- to 13-fold reduced affinities at recombinant as compared with native receptors (Fig. 6a; Table 1). For the $GABA_B$ receptor agonists GABA, APPA and L-baclofen, the rank order of affinities is identical at native and recombinant receptors (Fig. 6b). However, the affinities for these agonists at the recombinant receptors are reduced by 100-fold ($GABA_BR1a$) to 150-fold ($GABA_BR1b$) when compared to their affinities at rat cortex receptors (Table 1). In the case of the partial agonist CGP47656[42] a 30- and 50-fold lower affinity is found. The antagonists saclofen and 2-OH-saclofen may act as weak partial agonists[43] and therefore, similar to the agonists tested, show a difference in the ligand-displacement IC_{50} values at recombinant versus native receptors (Table 1). The Hill coefficients for full agonists are between 0.5 and 0.6 for native receptors and 0.7 and 0.9 for recombinant receptors (Table 1), suggesting multiple affinity states. The discrepancy in agonist binding affinity between native and recombinant receptors could be due to the presence of additional, pharmacologically distinct, $GABA_B$ receptor subtypes in the brain. However, alternative explanations are possible and are discussed below.

Based on the effectiveness of some ligands in preferentially modulating the release of one versus another neurotransmitter, the existence of auto- and heteroreceptor subtypes has been proposed[12–16,44]. The binding affinities of CGP35348 (Fig. 6a), CGP36742 and CGP52432 (data not shown), compounds reported to exhibit some subtype selectivity, were measured at $GABA_BR1a$ and -b receptors. The -b variant has slightly lower affinities for all agonists and antagonists tested, but otherwise no pharmacological difference was observed. This implies that only sequences common to both $GABA_BR1a$ and -b are directly involved in ligand binding.

$GABA_B$ receptors negatively couple to adenylyl cyclase

$GABA_B$ receptors are described to inhibit adenylyl cyclase activity, stimulate phospholipase A_2, activate K^+-channels, inactivate voltage-dependent Ca^{2+}-channels and modulate inositol phospholipid hydrolysis[41,45,46]. As $GABA_BR1a$ and -b have identical sequences in all domains predicted to be intracellular they are expected to be able to couple to the same effector systems. Using rat cortical slice preparations, L-baclofen was found to reduce forskolin-stimulated cAMP accumulation by about 40%[45]. We analysed the ability of $GABA_BR1a$ stably expressed in HEK293 cells to reduce forskolin-stimulated cAMP accumulation (Fig. 6c). We chose concentrations of forskolin and L-baclofen that should produce a maximal effect. Forskolin stimulates cAMP levels in HEK293 cells to more than 10 times the basal level. Stimulation of recombinantly expressed $GABA_B$ receptors by co-addition of 300 μM L-baclofen reduces forskolin-stimulated cAMP accumulation by about 30%. This inhibition is antagonized by CGP54626A, a $GABA_B$ receptor antagonist. The modulation of adenylyl cyclase activity by

$GABA_BR1a$ is sensitive to pertussis toxin, indicating that in HEK293 cells, which are deficient in G_o (ref. 47), $GABA_BR1$ couples to G_i. As a control, L-baclofen does not inhibit forskolin-stimulated cAMP formation in untransfected HEK293 cells (Fig. 6c). In preliminary experiments using $GABA_BR1a$, we were unable to detect positive coupling to adenylyl cyclase or coupling to the phospholipase C effector system. $GABA_BR1a/-b$ cRNA was injected into *Xenopus* oocytes, but no Ca^{2+}-dependent Cl^--currents or K^+-currents were detectable upon L-baclofen (100 μM) superfusion (J. Mosbacher, personal communication).

Discussion

Except for the $GABA_B$ receptors, the receptors for the major known neurotransmitters have been cloned. We have reported here the isolation of cDNAs encoding the $GABA_BR1a/-b$ proteins that exhibit many of the properties expected of $GABA_B$ receptors: (1) the amino-acid sequence is highly indicative of 7TM G-protein-coupled receptors and indicates a common gene family for the metabotropic receptors for GABA and L-glutamate; (2) when expressed in HEK293 cells, the cloned receptors negatively couple to adenylyl cyclase (Fig. 6c), as described for native $GABA_B$ receptors[45]; (3) recombinant $GABA_BR1a$ and -b proteins have similar M_r to $GABA_B$ proteins present in the nervous system of several vertebrate species (Figs 2d and 3c); and (4) the rank order of the binding activities of antagonists and full agonists are identical at recombinant and rat cerebral cortex $GABA_B$ receptors (Fig. 6a, b; Table 1). The above data suggest that the cloned receptors are the molecular correlates of the two $GABA_B$ receptors that we can distinguish in different brain structures.

Some findings, however, require further investigation. In contrast to antagonists, agonists have significantly lower binding activities at recombinant as opposed to native receptors (Fig. 6b; Table 1). No change in binding activity is observed when $GABA_BR1a$ and -b are coexpressed in COS-1 cells (IC_{50} values for GABA, APPA and L-baclofen are 18 μM, 3.2 μM and 29 μM, respectively), arguing against a heteromeric protein complex with increased agonist affinity. Low agonist affinity could be inherent to the cloned receptors, but it could also reflect properties of the heterologous expression system. For example, inefficient G-protein coupling or receptor desensitization could influence agonist affinity. It has been shown for $GABA_B$ receptors[20] and other G-protein-coupled receptors[48] that the binding affinity of agonist, but not of antagonists, is dependent on the G-protein coupling status. GTP, or its stable analogue Gpp(NH)p, reduces the affinity of native $GABA_B$ receptors to agonists by uncoupling the receptors from their G proteins. We determined that at cortex $GABA_B$ receptors the IC_{50} values for GABA, APPA and L-baclofen are ~10-fold higher higher in the presence of 300 μM Gpp(NH)p (data not shown). These IC_{50} values are still about 10-fold lower than the values obtained at recombinant receptors but indicate that G-protein coupling could at least in part account for the differences in agonist affinity observed. We did not detect any difference in agonist binding affinity between recombinant $GABA_B$ receptors expressed in COS-1 (Table 1) and HEK293 cells (Fig. 6a, b), even though in the latter the receptor is functionally coupled (Fig. 6c). However, in HEK293 cells only a fraction of the receptors might be coupled to G proteins, which could be the reason that a putative increase in agonist affinity was not measurable. Low- and high-affinity $GABA_B$ receptors have been described in functional assay systems[46]. In retrospect, should the cloned receptors represent a low-affinity $GABA_B$ receptor subtype, these receptors would not have been detected in previous autoradiographic studies, as only tritiated agonists were used to acquire such data[35,40]. This may explain some of the discrepancy found between the $GABA_B$ receptor *in situ* hybridization pattern described here (Fig. 5) and the distribution of binding sites reported earlier[39,40]. It has often been suggested that postsynaptic $GABA_B$ receptors are sensitive to PTX[4]. Although earlier experiments are not readily comparable to our experiments, the PTX sensitivity of the coupling of $GABA_BR1a$ in HEK293 cells (Fig. 6c) may indicate that this receptor could fulfil a postsynaptic role. As with the majority of neurotransmitter receptors, the cloning of additional $GABA_B$ receptors or the identification of more splice variants may explain the pharmacological differences reported previously. However, the receptors described here may also be able to couple to more than one G protein and multiple second messenger pathways and the pharmacological differences in functional assays may depend on the types and efficacies of effector systems available[45,49].

The ubiquitous distribution of $GABA_B$ receptors and their pharmacological actions suggest that they are likely targets in therapy, but their clinical importance awaits a deeper understanding of their molecular and functional diversity. The cloning of $GABA_B$ receptor cDNAs now provides the tools to study receptor heterogeneity and to correlate the known pre- and postsynaptic effects of $GABA_B$ receptors with distinct receptor molecules. □

Methods

Ligands. [^{125}I]CGP64213, the $GABA_B$ receptor antagonist used for expression cloning, and [^{125}I]CGP71872, the photoaffinity ligand used to tag $GABA_B$ receptor protein covalently, were synthesized from ethyl (1,1-diethoxyethyl) phosphinate[42]. Both ligands were labelled to a specific radioactivity of >2,000 Ci mmol^{-1} (ANAWA AG, Wangen, Switzerland). Experimental details on the syntheses of both ligands will be reported elsewhere. All the other ligands used in this study were synthesized in-house[3,24,42]. Their chemical structures are available as supplementary information.

Expression cloning of $GABA_BR1a$. Oligo (dT) primed double-stranded cDNA was synthesized from 5 μg poly(A)$^+$ RNA using a commercial cDNA synthesis system (Amersham, Superscript II reverse transcriptase from Gibco BRL). After the addition of *Bst*XI adaptors (Invitrogen), cDNAs >2 kb were gel-purified and ligated into pcDNAI (Invitrogen). Aliquots of the ligation mixture were transformed into electrocompetent MC1061/P3 *Escherichia coli* cells. Plasmid DNA was isolated from pools of 2,000 bacterial colonies obtained after the initial round of transformation. The DNA was introduced into COS-1 cells by DEAE-dextran transfection. COS-1 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM, 10% fetal calf serum, 15 μg ml^{-1} gentamycin). The cells were washed for 15 min in PBS, incubated for 9 min in 1 mg ml^{-1} (w/v) DEAE-dextran (Pharmacia) in PBS at room temperature, rinsed in PBS, and plasmid DNA was added (4 μg DNA in 540 μl PBS per 9 cm plate) and incubated for 30 min at 37 °C. Subsequently DMEM medium containing 10% NU-serum (Collaborative Research) and 80 μM chloroquine (Sigma) was added. After 4 h incubation at 37 °C the medium was removed and the cells were incubated for 2 min in 10% (vol/vol) dimethyl sulphoxide in PBS. The cells were rinsed in PBS, cell culture medium was added to the culture dishes and the cells were grown for an additional 2 to 3 days. COS-1 cells transfected with the plasmid pools were analysed for $GABA_B$ receptor expression using [^{125}I]CGP64213 binding. The cells were cooled on an ice bath, washed twice with ice-cold Krebs–Tris buffer (20 mM Tris-Cl pH 7.4, 118 mM NaCl, 5.6 mM glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$), and incubated for 80 min at room temperature with 0.2 nM of [^{125}I]CGP64213 in Krebs–Tris buffer. The cells were then cooled and washed twice with ice-cold Krebs–Tris buffer. Subsequently the dishes were air-dried and the walls of the plates were removed. Kodak X-OMAT AR films, together with intensifying screens, were exposed to the plates for 2 to 3 weeks at −80 °C.

Cross-hybridization screening. Bacterial colony hybridization was done using the ^{32}P-labelled $GABA_BR1a$ cDNA as probe (Boehringer Mannheim DNA labelling kit). Hybridization was carried out in 0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA at 60 °C and wash steps were with 0.5 × SSC, 0.1% SDS at 55 °C.

***In situ* hybridization and northern blot analysis.** *In situ* hybridization histochemistry using 10 μm coronal and horizontal cryosections (postfixed with 4% paraformaldehyde) of rat brain (male Tif RAI f [SPF] rats weighing 250 g) was as described[50]. ^{35}S-UTP/^{35}S-ATP-labelled riboprobes were generated from a cDNA fragment that is common to both $GABA_BR1a$ and -b. Post-hybridization was performed under high stringency conditions (63% forma-

mide, 80 °C for the post-hybridization wash). Slides were dipped into nuclear emulsion and exposed for 15 days. Northern blots were hybridized at 50 °C in the above solution containing 50% (vol/vol) formamide and filters washed with 0.1 × SSC, 0.1% SDS at 68 °C.

Ligand binding assay. Competition binding experiments were performed with COS-1, HEK293 or rat cortex cell membranes. To prepare membranes, cells were homogenized in Krebs–Tris buffer, centrifuged for 30 min at 40,000*g* and the pellet resuspended. Synaptic membranes were prepared as described[51]. Membranes were suspended in Krebs–Tris buffer at a concentration of approximately 50 μg ml^{-1} and incubated with 0.1 nM [^{125}I]CGP64213 for 90 min in the presence or absence of competitor ligands. The incubation was terminated by filtration through GF/C Whatman glass fibre filters.

Photoaffinity labelling. Cell membranes were incubated in the dark with 0.6 nM [^{125}I]CGP71872 for 1 h at room temperature. The incubation was terminated by centrifugation at 20,000*g* for 10 min at 4 °C. The pellet was washed in buffer to remove unbound from bound photoaffinity label, resuspended and illuminated with UV light (365 nm, 24 W) for 3 min. The suspension was centrifuged (20,000*g*, 20 min), the pellet washed in buffer and dissolved in SDS–PAGE loading buffer.

cAMP assay. The $GABA_BR1a$ cDNA was cloned into the vector pC1neo (Promega) and the plasmid transfected into HEK293 cells (ATCC). Stably expressing cell clones were identified after selection with G418 (1 mg ml^{-1}) using the [^{125}I]CGP64213 binding assay. The cells were grown to confluency on 15-cm tissue culture dishes. For cAMP-assays, cells were first washed, then detached with Krebs–Tris buffer containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) and subsequently incubated at 37 °C for 20 min. About 10^5 suspended cells were transferred to prewarmed (37 °C) tubes and 20 μM forskolin plus test agents were added for 20 min. The cells were collected by centrifugation and lysed by the addition of 1 ml 70% ethanol, 7 mM HCl. cAMP concentrations were measured using a kit (Amersham).

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